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ACYL COENZYME A: CHOLESTEROL ACYLTRANSFERASE (ACAT)

Government Support

The work leading to this invention was supported, in part, by research grants from The United States government.

Background of the Invention

Acyl coenzyme A: cholesterol acyltransferase (ACAT) is an intracellular enzyme that uses cholesterol and fatty acyl-coenzyme A (CoA) to form cholesterol esters. Accumulation of cholesterol esters as cytoplasmic lipid droplets within cells of human aortic tissue is a characteristic feature of early lesions of atherosclerotic plaque. In intestines of vertebrate animals, the extent of absorption of dietary cholesterol can be shown to be significantly reduced by inhibiting intestinal ACAT activity. In livers of vertebrate animals, formation of lipoproteins require proper supply of cholesterol esters produced through the ACAT catalyzed reaction.

ACAT is a membrane-bound enzyme located in the endoplasmic reticulum of various tissues of animal and human cells. The enzyme has been localized to the rough endoplasmic reticulum in rat liver. It is highly regulated in many cell types and tissues, and it is believed to play an important role in cholesterol metabolism in various cells and tissues such as the small intestinal mucosa, hepatocytes, macrophages, and the steroid hormone-producing tissues (O'Brien, P.M. and Sliskovic, D.R. (1992) in *Current Opinion in Therapeutic Patents*; Cadigan, K.M., et al. (1988) *J. Biol. Chem.* 263:274-282; Cadigan, K.M., et al. (1989) *J. Cell Biol.* 108:2201-2210).

Although ACAT has been studied intensively, much remains to be learned about its molecular structure. The active site of the enzyme has been localized to the cytoplasmic surface of the microsomal vesicles in the rat liver, using a combination of detergent and protease treatments, but whether the enzyme spans the entire membrane has not yet been determined. Lichtenstein, A.H. and Brecher, P. (1980) *J. Biol. Chem.* 255:9098-9104. Recent chemical modification studies indicate that essential histidyl and sulfhydryl residues may reside at or near the active site of the enzyme. Studies of ACAT activities of rabbit tissues suggest the existence of different ACAT subtypes since various tissues have

differing sensitivities to histidyl-modifying reagents. Kinnunen, P.M. et al. (1988) Biochemistry 27:7344-7350.

ACAT activity has been studied from ACAT solubilized and reconstituted from various cultured cells, including rat and pig liver cells. Although these procedures have allowed enzyme activity to be measured in a defined lipid environment, little progress has been made as yet in purifying the solubilized preparations. To date, no laboratory had succeeded in purifying ACAT to homogeneity with retention of biological activity.

Summary of the Invention

This invention pertains to purified, biologically active acyl coenzyme A:cholesterol acyltransferase (ACAT) and to nucleic acid (DNA or RNA) encoding acyl coenzyme A:cholesterol acyltransferase. The nucleic acid, or a fragment thereof, may be ligated with an expression vector and transfected into cells to express acyl coenzyme A:cholesterol acyltransferase activity in intact cells and in cell-free extracts. The nucleic acid, or fragments thereof, are useful as probes, as primers for polymerase chain reactions, or as antisense constructs.

Cells containing the nucleic acid, or active fragment thereof, as well as various cell-free systems are useful for screening and testing chemical agents serving as specific ACAT inhibitors. Such ACAT inhibitors are desirable in the development of drugs serving as specific ACAT inhibitors for prevention and/or treatment of various cholesterol-related disorders.

In addition, the nucleotide sequence of the gene encoding ACAT enables the screening of human populations for abnormal human ACAT activities for disease diagnosis. This invention provides a basis for creating various transgenic animals including mice and rabbits that permanently express the human ACAT gene. Such animals can be used to screen and test various agents that inhibit human ACAT activity in a tissue specific or non-tissue-specific manner in intact animals. In addition, this invention provides a basis for creating transgenic animals including chickens, cows and pigs with permanently reduced ACAT activity. Animals with lower ACAT activity have much less body cholesterol ester content, and thus would offer the same nutritional value but with less dietary cholesterol intake to consumers.

Brief Description of the Drawings

Figure 1. Southern analysis of enzyme restricted genomic DNAs probed with ^{32}P -gDNA G. Genomic DNAs were from 25-RA (lane 1), AC29 (lane 2), 29T2-8 Amph^R 4,6,8,10,11,12,16 (lanes 3-9), 29T1 (lane 10), 29T2-4 (lane 11), 29T2-8 (lane 12), 29T2-10 (lane 13), human fibroblast (lane 15). Genomic DNAs were digested with EcoRI and Hind III, run on a 0.8% agarose gel, transferred to a nylon filter and probed with radiolabeled gDNA G. Fifteen μg of genomic DNA was used for each sample except for human fibroblasts (5mg). Lane 14 contains 10 μg of λ DNA (Hind III cut) as a size marker.

Figure 2. Northern analysis of polyA⁺ mRNAs probed with ^{32}P -gDNA G₂. PolyA⁺ mRNAs were prepared using FAST-TRACK (Invitrogen, Inc.) from confluent monolayer cells grown in media with 10% fetal calf serum of AC29 (lane 1), 25-RA (lane 2), T2-8 Amph^R4 (lane 3), T2-8 Amph^R10 (lane 4), T2-4, 8, 10 (lanes 5-7), and human A431 cells (lane 8). RNAs were run on a denaturing gel and blotted onto a nylon filter, cross-linked with UV light. Approx. 15 μg of RNA was used per lane. A control experiment showed that the same blot probed with ^{32}P -actin cDNA provided a strong and sharp signal at the 1.9 kb region with approximately equal intensity for all 8 lanes.

Figure 3. Nucleotide sequence of cDNA C₁, as determined by double stranded DNA sequencing.

Figure 4. Southern analysis of enzyme restricted genomic DNAs probed with ^{32}P -cDNA C₁. Genomic DNAs were from 25-RA (lane 2), AC29 (lane 3), T2-8 Amph^R4, 6, 8, 10, 11, 12, 16, 17, 18 (lanes 4-8, 10-13), 29T1 (lane 9), 29T2-4, 8, 10 (lanes 14, 15, 16), human fibroblast (lane 17). Lane 1 contains 10 μg of gDNA (Hind III cut) as a size marker. Genomic DNAs were digested and analyzed in the same manner as described in Fig. 2, except the ^{32}P -probe was cDNA C₁.

Figure 5. Northern analysis of polyA⁺ mRNAs probed with ^{32}P -cDNA C₁. A duplicate blot prepared in an identical manner as described in Fig. 2 was probed with either ^{32}P -cDNA C₁ (A), or ^{32}P -actin cDNA (B).

Figure 6. The nucleotide sequence of cDNA K₁. The region which overlaps with that of cDNA C₁ is underlined.

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Figure 7. 25-RA cells (A), AC29 cells (C) the stable transfectant cells 29 K₁-14e treated with (D) or without (B) ACAT inhibitor 58-035 viewed with differential-interference contrast microscopy. Cells were plated and processed for differential-interference contrast microscopic viewing by the same procedure as described in Cadigan, K.M., et al. (1989) J. Cell Biol. 108:2201-2210. In (D), cells were treated with 58-035 at 400 ng/ml for 36 h.

Figure 8. Heat inactivation of reconstituted ACAT activity from 25-RA (symbol = open square), 29 T2-8 (symbol = closed diamond), 29 K₁-4b (symbol = closed square), and 29 K₁-14e (symbol = partially open diamond). Cells were grown in 162 cm² flasks in medium A to confluence. They were harvested, and the cell extracts were reconstituted according to the procedure of Cadigan and Chang (1988) J. Lipid Res. 29:1683-1692. The reconstituted samples were incubated at 45°C at indicated times, then placed on ice prior to assay for enzyme activity. The control activities for 25-RA, 29 T2-8, 29 K₁-4b, and 29 K₁-14e were 228, 73, 43, and 109 pmoles/min per mg respectively.

Figure 9. The nucleotide and predicted amino acid sequences of cDNA K₁. Nucleotide residues are numbered on the right; amino acid residues are numbered on the left with residue 1 being the putative initiator methionine. The 5 stretches of sequences sharing significant homology with firefly luciferase "signature sequences" regions 1, 2 or 3 (Babbitt et al., (1992) Biochemistry 31: 5594-5604) are underlined in the protein coding region. Leucines involved in the potential leucine heptad motif are identified by asterisks. The potential N-linked glycosylation site is indicated by a double asterisk (amino acid residue 409). The two AATAAA sites are underlined in the 3' - untranslated region.

Detailed Description

The enzyme acyl coenzyme A:cholesterol acyltransferase (ACAT) is an intracellular enzyme which previously had not been purified to homogeneity with retention of biological activity. This invention pertains to isolated, biologically active acyl coenzyme A:cholesterol transferase, or a biologically active portion thereof. As used herein, biological activity includes catalytic activity. ACAT has been shown to have amino acid sequences TNLIEKSASLDNGGCALTT, GRLVLEFSLLSYAF, GFGPTY, GYVAMKFAQVFGCF, and ARVLVLCUFNSILPGVL, as shown in Sequence Listing Nos. 5 - 9, respectively, and their functional equivalents, which are believed to

be involved in catalytic activity. The enzyme, or active portion, is preferably human in origin.

The invention also pertains to the nucleic acid (DNA or RNA) encoding acyl coenzyme A:cholesterol acyltransferase and to the use of the nucleic acid to produce, by recombinant techniques, acyl coenzyme A:cholesterol acyltransferase.

One embodiment of the invention is the cDNA for human ACAT contained the clone K₁, or any derivative of this cDNA. This nucleotide sequence is shown in Sequence Listing No. 2. Variants of this ACAT nucleotide sequence are also within the scope of this invention. These include sequences substantially homologous to the sequence of Sequence Listing No. 2. This includes sequences, such as those derived by mutagenesis, which have nucleotide insertions, deletions, substitutions, or other modifications, but which encode a catalytically active ACAT. The variants include fragments of the ACAT nucleotide sequence. As used herein, a fragment of the nucleotide sequence encoding human acyl coenzyme A:cholesterol acyltransferase refers to a nucleotide sequence having fewer nucleotides than the nucleotide sequence of the entire enzyme. Nucleic acid sequences used in any embodiment of this invention can be cDNA as described herein, or alternatively, can be any oligonucleotide sequence having all or a portion of a sequence represented herein, or their functional equivalents. Such oligonucleotide sequences can be produced chemically or mechanically using known techniques. A functional equivalent of an oligonucleotide sequence is one which is capable of hybridizing to a complementary oligonucleotide to which the sequences shown in the Sequence Listing, or fragment thereof, hybridizes, or a sequence complementary to either of the sequences shown in the Sequence Listing.

ACAT, or a portion of ACAT, can be produced by standard recombinant techniques using the nucleotide sequences of this invention. The nucleotide sequence encoding ACAT is inserted into an expression vector. A suitable host cell, such as a mammalian cell, is transformed with the vector, and the cell is cultured under conditions conducive to the production of the enzyme by the cell. ACAT, or a portion of ACAT, can be produced in other organisms, including bacteria, yeast, and insect cells, as well as various cell-free systems. A portion of ACAT expressed in these systems may express partial ACAT function, such as the ability to bind, inter alia, cholesterol, fatty acids, and coenzyme A, thus

creating unique tools and assays for testing and screening for inhibitors which block these partial ACAT functions. these inhibitors would be genuine ACAT inhibitors.

The nucleotide sequence information contained in the cDNA encoding ACAT also provides crucial information concerning the catalytic mechanism of ACAT and provides investigators with a means for rational design of drugs serving as specific ACAT inhibitors. Such ACAT inhibitors are desirable for prevention and/or treatment of human hypercholesterolemia and human atherosclerosis. The nucleotide sequence information contained in the nucleic acid encoding ACAT enables design of various specific oligonucleotides as specific anti-sense DNAs or anti-sense RNAs, to inhibit human ACAT messenger RNAs, thereby to inhibit ACAT protein production, as described in more detail below.

The nucleic acid molecules of this invention can be used to produce primers for polymerase-mediated replication of nucleotide sequences encoding ACAT. Typically, the primer is a single stranded oligonucleotide substantially complementary to a portion of the ACAT sequence to be replicated. The primer will have a length sufficient to prime polymerase activity, generally a minimum of five to seven nucleotides, and typically from 16 to 30 nucleotides. Primers can be used in polymerase chain reaction (PCR) to amplify ACAT nucleotide sequences.

The nucleic acid molecules of this invention, and fragments thereof, are also useful as hybridization probes for library screenings to isolate and identify partial and/or full length cDNA or gDNA clones encoding ACAT genes from various animal species. Probes are generally labeled single stranded oligonucleotides substantially complementary to at least a portion of the ACAT nucleotide sequence. Hybridization reactions can be performed by standard techniques. Such probes can be used to identify different forms of human ACAT or ACAT from different animal species.

The probes and primers described above are useful as diagnostic tools to identify persons who have certain diseases, either acquired or genetically inherited, related to an abnormality in the ACAT gene or gene expression.

Nucleic acid molecules can be used to produce antisense constructs for inhibition of ACAT activity. In one embodiment, the oligonucleotide is an

antisense oligonucleotide. The antisense oligonucleotide can be a normal oligonucleotide for an analogue of an oligonucleotide (e.g., phosphorothioate oligonucleotides, in which one of the phosphate oxygens is replaced by a sulfur atom) sufficiently stable to reach the target in effective concentrations. Antisense oligodeoxynucleotides can be prepared by standard synthetic procedures.

In another embodiment, the antisense construct is oligoribonucleotide. The antisense construct is produced by introducing the gene encoding the construct into a cell. For example, an ACAT nucleotide sequence can be placed in an expression vector in reverse orientation to generate an antisense transcript.

The antisense oligonucleotides can be designed to operate by different mechanisms of gene inhibition. Generally, these mechanisms involve the hybridization of the oligonucleotide to a specific RNA sequence, typically a messenger RNA. The targeted sequence can be located in the coding region of the RNA or it can be a signal sequence required for processing or translation of the RNA. Alternatively, the oligonucleotide may form a triple helix DNA structure, inhibiting transcription of the mRNA sequence.

The nucleic acid sequence of this invention can be used to produce transgenic animals either carrying human ACAT or having reduced levels of ACAT activity. Transgenic mammals, such as mice, expressing full or partial human ACAT activity can be easily created by methods well-documented in the art, for example those described in Leder et al., U.S. Patent No. 4,736,866. One of ordinary skill in the art can prepare transgenic mammals by injecting the ACAT gene, or a portion thereof, into the germline of the mammal. Alternatively, the gene or gene fragment can be injected into the male pronucleus of the fertilized egg when the egg is at the single cell stage, prior to implanting the egg in the host female. Moreover, using similar methods, a transgenic animal, such as a chicken, cow, or pig, can be produced by, for example, transfecting germ cells with a nucleic acid sequence encoding an antisense construct which blocks ACAT expression. Transgenic mammals carrying those constructs would have decreased ACAT activity, and, as a result, lower body cholesterol levels. Such transgenic animals would offer the same nutritional values while decreasing consumers' dietary cholesterol intake.

The invention further comprises a stable mutant cell which lacks endogenous ACAT activity, and is transformed with a nucleic acid encoding

human ACAT, such that the cell expresses activity of human ACAT, preferably at high levels, in intact cells and in cell-free extracts. The cell produces an excess of cholesterol ester, causing the cell to form detectable (e.g. visibly) cytoplasmic lipid droplets. These droplets disappear with inhibition of ACAT. This mutant cell containing the human ACAT gene can be used in an assay for agents, including antisense DNA and/or RNA, that inhibit human ACAT activity. The cell is exposed to the agent under conditions which allow the agent to be taken up into the cell, and the cell is examined for substantial disappearance of the lipid droplets. Substantial disappearance indicates inhibition of human acyl coenzyme A:cholesterol transferase. This invention also embraces any agents which inhibit ACAT identified by the above-described screening assay, or any other assay using the ACAT nucleic acid sequence, or fragments thereof.

The invention is illustrated further by the following exemplification.

EXEMPLIFICATION

Example I.

A. Preparing Human ACAT Genomic DNA Fragments

Chinese hamster ovary (CHO) cells are a fibroblast-like cell line in which cholesterol ester synthesis is highly regulated by exogenous sources of cholesterol, such as low density lipoprotein (LDL), and by endogenous cholesterol synthesis. The inventor and others previously developed an amphotericin B enrichment procedure, and reported the isolation of CHO cell mutants almost entirely lacking ACAT activity. All isolated mutants were found to belong to the same complementation group and possess a defect in the ACAT enzyme itself or in a factor needed for production of the enzyme (Cadigan, K.M., et al. (1988) J. Biol. Chem. 263:274-282).

Cells that regained the ability to synthesize cholesterol esters were isolated from the mutants described above. After populations of ACAT deficient mutant (AC29) were subjected to chemical mutagenesis, or transfected with human fibroblast whole genomic DNA, two revertants and one primary transfectant (T₁) were isolated. Isolation was achieved by virtue of the revertant cells' or transfectant cells' higher fluorescent intensities when stained with Nile Red, a stain specific for neutral lipids, including cholesterol esters.

Both revertants and transfectants regained large amounts of intracellular cholesterol ester and ACAT activity. However, heat inactivation experiments reveal that the enzyme activity of the transfectant cells has heat stability properties identical to those of human fibroblasts, while the ACAT activities of the revertants are similar to that of other Chinese hamster ovary cell lines. This demonstrates that the molecular lesion in the ACAT deficient mutants resides in the structural gene for the enzyme, and indicates that the transfectant cells corrected this lesion by acquiring and stably expressing a human gene encoding the human ACAT polypeptide.

Secondary transfectants (T2-4, T2-8, and T2-10) were isolated by transfection of ACAT deficient mutant cells with primary transfectant genomic DNA. Genomic Southern analysis of the secondary transfectants, using a probe specific for human DNA, revealed several distinct restriction fragments common to all the transfectants. These fragments were hypothesized to comprise part or all of the human ACAT gene (Cadigan, K.M., et al. (1989) J. Cell Biol. 108:2201-2210). These human gene fragments were isolated (see Section B below) and were used as the starting material for molecular cloning of the human ACAT cDNA of this invention.

Standard recombinant DNA techniques were employed, according to the methods known in the art and as described in Sambrook, J., et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. The use of λ ZAP, λ DASH, pBluescript for library or recombinant plasmid constructions were following instructions manuals provided by Stratagene, Inc. The use of FAST-TRACK kit for mRNA isolation, of pcDNA₁ and pcDNA_{neo} for library or recombinant plasmid constructions were following instruction manuals provided by Invitrogen Inc. Other biochemical assays and methods used here were all previously documented in Cadigan, K.M., et al. (1988) J. Biol. Chem. 263:274-282; Cadigan, K.M., et al. (1989) J. Cell Biol. 108:2201-2210; Hasan, M.T., et al. (1991) Somatic Cell and Mol. Genetics 17:413-517; Cadigan, K.M. and Chang, T.Y. (1988) J. Lipid Res. 29:1683-1692.

B. Isolating human ACAT cDNA C₁ Clone

A phage lambda (λ DASH, from Stratagene, Inc.) library consisting of genomic DNA fragments of transfectant cell T2-8 was prepared and screened

using the human-specific Alu-repetitive DNA as the probe prepared according to the method of Cadigan, K.M., et al. (1989) J. Cell Biol. 108:2201-2210. An Alu-positive, λ clone (designated as λ G) containing an insert was isolated. The insert, designated as gDNA G, was determined to be approximately 14 kb in length. Insert gDNA G was labeled with ^{32}P and used as the probe in genomic Southern analyses of restriction-digested genomic DNAs of human skin fibroblasts, primary transfectant cell clone T₁, and of secondary transfectant cell clones T2-4, T2-8, and T2-10. Results (Fig. 1) show gDNA G is a specific, common-sized human DNA fragment present in the genomes of all four transfectant clones which exhibit human ACAT activity (Cadigan, K.M., et al. (1989) J. Cell Biol. 108:2201-2210). Fragment gDNA G was not found in the genomes of 25-RA cells or AC29 cells, which suggests that gDNA G may be part of the human ACAT genomic DNA.

Table 1
[^3H] Oleate Incorporated Into Cholesteryl
Oleate in Intact Cells
(% of 25RA)

| Cell Type | (% of 25RA) |
|---------------------------|-------------|
| 25-RA | 100.0* |
| 29T2-8 | 94.5 |
| 29T2-8Amph ^{R4} | 1.7 |
| 29T2-8Amph ^{R6} | 0.5 |
| 29T2-8Amph ^{R8} | 1.1 |
| 29T2-8Amph ^{R10} | 0.8 |
| 29T2-8Amph ^{R11} | 0.6 |
| 29T2-8Amph ^{R12} | 0.0 |
| 29T2-8Amph ^{R16} | 0.9 |
| 29T2-8Amph ^{R17} | 1.1 |
| 29T2-8Amph ^{R18} | 1.2 |

*100% = 7529 dpm/min/mg

To demonstrate the accuracy of this theory, nine individual ACAT deficient cells were isolated using the secondary transfectant cell T2-8 as the parental cell. The T2-8 cell was found to be very sensitive to amphotericin B killing. Using the same procedure as previously described for isolating ACAT deficient mutants from 25-RA cells (Cadigan, K.M., et al. (1988) J. Biol. Chem. 263:274-282), nine independent cell clones (designated as T2-8 Ampho R4, T2-8 Ampho. R6; etc.) were obtained from approximately 10×10^6 T2-8 cells. These clones are found to be devoid of ACAT activity when analyzed by ^3H -oleate pulse in intact cells, as shown in Table 1.

Southern analyses (Fig. 1, lanes 3-9) using gDNA G as the probe showed that, in contrast to the parental T2-8 cells, none of these independent cell clones contains DNA fragment G as part of their genomes. This demonstrates 100% concordance between the presence/absence of DNA fragment G in the cell genome and the presence/absence of human ACAT activity in various CHO cells, and strongly supports the proposition that gDNA G is part of the human ACAT genomic DNA.

gDNA fragment G was digested with *Hin*fl. The resulting 1.2 kb fragment designated as gDNA G₂, which was found to be devoid of Alu-repetitive DNA, was isolated and cloned into the phagemid vector pBluescript (Stratagene). Fragment gDNA G₂ was found to contain at least one exonic element, since it strongly hybridized (particularly at the 3.8 kb and 3.0 kb regions) with polyA⁺ mRNAs of discrete sizes prepared from all of the human ACAT positive transfectant cells, and from human epidermal carcinoma A431 cells. The results of the Northern analysis of those polyA⁺ mRNAs is shown in Figure 2.

A cDNA library was prepared using polyA⁺ mRNAs of T2-8 cells using pcDNA₁ (from Invitrogen, Inc.) as the vector. This library was screened with ³²P gDNA G₂. A single cDNA clone (size = 1006 bps) was identified, designated as cDNA C₁, and sequenced. As shown in Figure 3 and the Sequence Listing, the nucleotide sequence contains a single, uninterrupted open-reading frame for a predicted polypeptide of 335 amino acids. Extensive search for nucleotide sequence homology between C₁ DNA and other DNAs of known sequences in several DNA sequence data banks reveals that the C₁ sequence has never been reported in the art. Genomic Southern analyses, Figure 4, show that ³²P-C₁ DNA strongly hybridizes with the same 14-kb genomic DNA fragment recognized by gDNA G in all the transfectant cell DNAs, and in human fibroblast DNAs. Northern analyses, shown in Figure 5, demonstrate that ³²P-C₁ DNA strongly hybridizes with the same polyA⁺ mRNA species as recognized by the gDNA G₂ fragment in transfectant cell RNAs. These hybridization signals were absent in CHO cells devoid of human ACAT activity (Fig. 4, lanes 2-8 and 10-13; Fig. 5, lanes 1-4), consistent with the interpretation that C₁ DNA is part of the human ACAT cDNA. Clone C₁ DNA was ligated in two opposite orientations with the mammalian expression vector pcDNA_{neo} (from Invitrogen), and then transfected into AC29 cells. These experiments repeatedly failed to produce functional complementation of ACAT deficiency in AC29 cells, thus indicating that C₁

DNA does not contain sufficient coding sequences to express human ACAT activity in CHO cells.

Example II. Isolating Human ACAT cDNA K₁ Clone

A phage lambda library (in λ ZAP; Stratagene) containing cDNAs of human macrophage cell line THP-1 cells was obtained from Dr. T. Kodama of Tokyo University in Japan as a generous gift. (Preparation and use of this particular library is described in Matsumoto, et al. (1990) *Proc. Natl. Acad. Sci.* 87:9133-9137). This library was screened using both gDNA G₂ and cDNA C₁ as the probe. A single clone was identified which strongly hybridizes with both G₂ and C₁ probes. This clone, designated as cDNA K₁, is approximately 4.1 kb in length.

The entire K₁ nucleotide sequence has been completed, with 98% to 99% certainty, and is shown in Figure 6 and in the Sequence Listing. Uncertain nucleotides are represented by the letter N. The K₁ nucleotide comprises a 1006-bp nucleotide sequence (underlined) which shares 100% homology with that of the DNA C₁ sequence shown in Figure 3. K₁ cDNA can be stably propagated as an insert in the phagemid pBluescript.

To demonstrate that K₁ DNA complements ACAT deficiency in AC29 cells, the pBluescript plasmid containing K₁ DNA as the insert (designated as pK₁) was digested with enzymes NotI and EcoRV, to release the intact K₁ DNA insert free of NotI and EcoRV sites from the vector. The DNA mixture was ligated with a NotI-EcoRV linearized pcDNA₁ vector for the purpose of ligating K₁ DNA with the CMV promoter in proper orientation. The ligated DNA mixture was directly transfected into AC29 cells. Appropriate control transfections, using various DNA mixtures without K₁ DNA or without pcDNA₁ were performed in parallel. The result (Table 2) indicates that DNA K₁ is necessary to provide large increases in rate of cholesteryl ester synthesis in AC29 cells, in both transient and stable transfection experiments.

Table 2

Transfection of Various DNA Mixtures Into ACAT Deficient Mutant (Clone AC29)

Relative Rate of
Cholesterol Ester

Relative Rate of

| DNA Mixture | Synthesis In Intact Cells | | Phospholipid Synthesis In Intact Cells |
|--|---------------------------|------------------------|--|
| | A. Transient Transfection | B. Stable Transfection | |
| None | 1.0* | 1.0** | 1.0*** |
| pSV2 neo | 1.0 | 1.5 | 1.2 |
| pSV2 neo+pBluescript | 0.9 | 1.3 | 1.7 |
| pSV2 neo+pcDNA ₁ | 1.1 | 1.6 | 0.9 |
| pSV2 neo+pBluescript +pcDNA ₁ | 1.0 | 1.0 | 1.6 |
| pSV2 neo+pBluescript +K ₁ | 2.4 | 8.6 | 1.4 |
| pSV2 neo+pBluescript +pcDNA ₁ +K ₁ | 6.0 | 21.8**** | 1.0 |

* 1.0 = 134 dpm/min/mg

** 1.0 = 80 dpm/min/mg

*** 1.0 = 23×10^2 dpm/min/mg; measured only in stable transfectant cells

**** 12.4% of value found in 25-RA cells

In the experiments reported in Table 2, DNA transfection of AC29 cells was accomplished according to the method of Hasan et al. ((1991) Somatic Cell and Mol. Genetics 17:413-517). AC29 cells plated at 0.3×10^6 cells/25 cm² flask in medium A were grown for 24 h before transfection. Each transfection was performed in triplicate, and included supercoiled plasmid pSV2_{neo} (at 0.7 µg/flask) along with indicated DNA mixtures (which totaled 17.5 µg/flask). Each indicated plasmid was sequentially cut with EcoRV and NotI, salt precipitated, and redissolved in sterile water.

For the DNA mixture involving pBluescript + pcDNA₁, or pBluescript + pcDNA₁ + K₁, ligation took place as follows: 50 µg pcDNA₁ was ligated with

either 12.5 µg pBluescript, or with 25 µg pK₁ (cut with EcoRV and NotI to release K₁ insert from vector) in 20 µl volume using 3400 units of T4 DNA ligase (New England Biolab) at 16°C overnight. The ligated DNA mixtures were salt precipitated, redissolved in sterile water and used directly in transfection experiments.

To measure cholesterol ester synthesis in transient transfectant cells, transfected cells were grown in medium A for 2 days, then in medium A + 500 µg/ml G418 for one more day, and were subjected to ³H-oleate pulse assay in duplicate flasks. To measure cholesterol ester synthesis in stable transfectant cells, cells after transfection were grown in medium A for 2 days, then in medium A + 500 µg/ml G418 for 14 days. The G418 resistant cells were then placed in medium A in duplicate flasks, and were subjected to ³H-oleate pulse assay.

In a separate experiment, DNA mixtures of pBluescript + pcDNA_{neo}, or of pBluescript + pcDNA_{neo} + K₁ were treated, ligated, and used for stable transfection in an identical manner to that described in Table 2. Stable transfectant cells (resistant to 500 µg/ml G418 toxicity) were isolated and subjected to ³H-oleate pulse assay. Results very similar to those shown in Table 2 were obtained: While the transfectant clones resulting from the former DNA mixture only provided basal values, those cells resulting from the latter DNA mixture provided large increase (by approximately 10-fold) in rate of cholesterol ester synthesis as compared to the basal value found in AC29 cells.

In the stable transfectant cell populations containing pcDNA₁ and K₁ DNA, or containing pcDNA_{neo} and K₁ DNA, a great deal of heterogeneity was observed in cytoplasmic cholesteryl ester contents, present as lipid droplets, in various cell clones. This can be visually detected by examination of cells under phase-contrast microscopy. That this is so appears to be due to variability of expression of the transfected K₁ gene in different clones.

Example III: Stable Transfectant 14e

The stably transfected cells described above were cloned by cloning rings. Eight independently cloned transfectant cells were evaluated for their rates of cholesterol ester synthesis in intact cells and in vitro by reconstituted ACAT assay. The result (shown in Table 3) indicates that one clone, identified as 14e,

expresses the highest ACAT activity in intact cells and *in vitro*. Its ACAT activity is higher than those found in the transfectant clone T2-8 obtained previously through total human genomic DNA transfection experiments. A second stable transfectant clone (4b), obtained using the ligated DNA mixture of pcDNA_{neo} + pBluescript + K₁, expresses significant ACAT activity, but this activity is less than that measured in the T2-8 cells.

Table 3

Rates of Cholesterol Ester Synthesis of Individual AC29 Clones Stably Transfected with K1 cDNA

| Cell Type | In Intact Cells (by Oleate Pulse) | <u>In Vitro</u> (By Reconstituted ACAT Assay) |
|-----------|--------------------------------------|--|
| AC29 | 1.0* | 1.0** |
| 29K1-10 | 0.7 | 1.0 |
| 29K1-11 | 1.1 | 1.0 |
| 29K1-12 | 0.8 | 1.1 |
| 29K1-6 | 1.1 | 1.9 |
| 29K1-13 | 5.4 | 3.1 |
| 29K1-5 | 0.9 | 4.3 |
| 29K1-4b | 42.4 | 13.6 |
| 29K1-14c | 82.4 | 23.3 |
| 29T2-8 | 70.6 | 16.1 |
| 25-RA | 84.2 | 44.4 |

* 1.0 = 133 dpm/min/mg

** 1.0 = 4 pmole/min/mg

In the experiments reported in Table 3, Clones 29K₁-10, 11, 12, 13, and 14e were isolated from stable transfectant cells described in Table 2 using pSV2_{neo} + pBluescript + K₁ as the DNA mixture; clones 29 K₁-4b, 29 K₁-5, 29 K₁-6 were isolated from stable transfectant cells using pBluescript + pcDNA_{neo} + K₁, performed in a separate experiment in similar manner as described in Table 2; clones 14a, 14d, and 14e were isolated from stable transfectant cells described in Table 2 using pSV2_{neo} + pBluescript + pcDNA₁ + K₁ as the DNA mixture. The oleate pulse assay and in vitro reconstituted ACAT activity assay were performed in duplicate as described earlier (Cadigan, K.M., et al. (1988) J. Biol. Chem. 263:274-282; Cadigan, K.M., et al. (1989) J. Cell Biol. 108:2201-2210).

In 14e cells, numerous cytoplasmic lipid droplets are visible under the microscope (Fig. 7B). When treated with an ACAT inhibitor, specifically 58-035 at 400 ng/ml for 36 h, most of the lipid droplets in 14e cells disappear (Fig. 7D), indicating that these are cholesteryl ester droplets. For comparison purposes, photos of 25-RA cells, which contain ACAT of CHO origin, and AC29 cells, which are deficient in ACAT activity, as viewed under the microscope, are provided in Fig. 7A and 7C. The cloned populations of 14e cells can be continuously grown in culture for at least two months without losing this distinct phenotype.

As was previously reported, the biochemical characteristics of ACAT activities present in the crude extracts of cultured human cells differs from that in CHO cells. Cadigan, K.M. et al. (1989) J. Cell Biol. 108:2201-2210: In reconstituted vesicles of defined lipid composition, the CHO cell ACAT activity exhibits a significantly greater thermolability at 45°C than that of human cell ACAT activity. Based on this criterion, primary and secondary genomic ACAT transfectant cells (29T1, 29T2-4, 29T2-8, and 29T2-10) were determined to contain ACAT activities of human origin. Further investigation, by heat inactivation of the ACAT activities expressed in stable cDNA K₁ transfectant clones 14e and 4b, and comparison with that expressed in 25-RA cells and in T2-8 cells shows that the ACAT inactivation rates in 14e cells and 4b cells are the same as that of T2-8 cells, which is considerably slower than that found in 25-RA cells. This indicates that the ACAT activities expressed in 14_e cells and 4_b cells are of human origin. This result invalidates the alternative interpretation: that the K₁ cDNA was human cDNA which, upon transfection in AC29 cells, reactivated the CHO ACAT activity. If this were the case, the ACAT activity expressed in cells 14e and 4b would have behaved like that expressed in 25-RA cells, i.e., the CHO ACAT, in the heat inactivation study.

This cell clone can effectively be used as a tool to screen drugs and antisense constructs serving as human ACAT inhibitors. The numerous cytoplasmic lipid droplets in 14e cells that are visible under the microscope provide an elegant test for evaluating potential ACAT inhibitors. Specifically, when 14e cells are treated with an ACAT inhibitor, the lipid droplets essentially disappear, as illustrated in Figure 7D. A simple, visual method for testing and screening potential human ACAT inhibitors in cultured cells is thus provided. Those skilled in the art will recognize that this embodiment is not limited to 14e cells, and can be used with any stable transfectant cell line that hyper expresses

the ACAT gene, or a fragment thereof, for example, the 29K-4b or 29T₂ cell lines. Those skilled in the art will also recognize that the visual detection of intracellular cholesteryl esters present in 14e cells, or other cell line capable of hyper expressing ACAT, could be achieved by means other than standard microscopy, such as phase-contrast microscopy, fluorescent dye staining followed by fluorescent microscopy, among others. The speed of detection may also be enhanced by coupling a rapid scanning mechanism to the microscopic apparatus.

Example IV. 1.7 kb K₁ cDNA Encoding Human ACAT

A fragment of the 4.0 kb K₁ cDNA was discovered that spans the entire predicted protein coding region of ACAT. It is the 1.7 kb Sal I - Hind III fragment, spanning nucleotide residues 1302-3050 of K₁.

Subcloning the 1.7 kb fragment into the pcDNA1_{neo} vector, in both directions, produced plasmids designated pcDNA1_{neo} - K_{1.7kb sense} and pcDNA1_{neo} - K_{1.7kb antisense}. To demonstrate ACAT expression, the plasmids, together with pcDNA_{neo} as a control, were transiently transfected into AC29 cells. As shown in Table 4, transfection of pcDNA1_{neo} - K_{1.7kb sense} dramatically increased the rate of cholesterol ester synthesis in AC29 cells, with values equal to 60% of those found in 25-RA cells. The plasmid minimally increased the rate of phospholipid synthesis. Control plasmids exhibited no similar effects. Plasmid pcDNA1_{neo} - K_{1.7kb sense} also increased the rate of cholesterol ester synthesis in stable transfectant cells, approximately 20% of values found in 25-RA cells, without altering the rates of phospholipid synthesis.

Table 4

Transient Transfection of Plasmids Containing pcDNA1_{neo} Vector and K1 1.7kb cDNA as Insert into ACAT Deficient Mutant (Clone AC29)

| DNA Mixture | Relative Rate of Cholesterol Ester Synthesis In Intact Cells | | Relative Rate of Phospholipid Synthesis In Intact Cells | |
|---|--|---------------------------------|---|---------------------------------|
| | A. Third day after Transfection | B. Fifth Day after Transfection | A. Third day after Transfection | B. Fifth day after Transfection |
| pcDNA1 _{neo} | 1.0 ^a | 1.0 ^b | 1.0 ^c | 1.0 ^d |
| pcDNA1 _{neo} -K1 1.7kb (antisense) | 0.9 | .07 | 1.0 | 1.0 |

| | | | | |
|--|-----|-----|-----|-----|
| pcDNA1 _{neo} -K1 1.7kb (sense) | 103 | 91* | 1.7 | 1.4 |
|--|-----|-----|-----|-----|

| | |
|---|-----------------------|
| a | 1.0 = 29 dpm/min/mg |
| b | 1.0 = 45 dmp/min/mg |
| c | 1.0 = 1404 dpm/min/mg |
| d | 1.0 = 164 dpm/min/mg |

* 60.5% of value found in 25-RA

The method of Hason et al. ((1991) Somatic Cell and Mol. Genetics 17:413-417) was used to perform transfection. 0.3×10^6 cells per 25 cm² flask were seeded in medium A for 24 h. 3 ml of fresh medium A with 100 μ M Chloroquine was then added for 2 h before the transfection. For each flask, 3 μ g of pcDNA1_{neo} DNA or 5 μ g of pcDNA1_{neo}-K1 1.7kb DNA was used in transfection. Incubation was at 37°C for 16 h. Transfection cells were grown in medium A +500 μ g.ml G418 for 3 or 5 days and were then subjected to ³H-oleate pulse assay in duplicate flasks. The construction of pcDNA1_{neo}-K1 1.7kb plasmids was described in Experimental Procedures.

As shown in Fig 4., the K1 cDNA contains a single open reading frame (ORF) (residues 1397-3046) 1650 bps in length and a predicted 64,805 dalton protein. This ORF is designated as ACAT K1 protein. The second and third nucleotides before the putative first ATG codon and the one after it conformed to the Kozak sequences (Kozak, 1984). An in-frame stop codon was found 150 nucleotides upstream from the first ATG codon.

Hydrophobicity analysis of the hypothetical ACAT K1 protein indicates that it contains at least two potential transmembrane α -helices located at amino acids 132-155 and 460-483 (Fig. 7). This analysis supports the conclusion that ACAT K1 is an integral membrane protein. The polypeptide regions at amino acids 215-235, 320-340, and 355-380 are also very hydrophobic, yet these regions seem to be rich in β -sheet structure (panel B of Fig. 7), therefore, these regions may not contain transmembrane helices. One potential N-glycosylation site (Gavel and von Heijne, (1990) Protein Engineering 3:433-442) was identified (indicated by the symbol ** in Fig. 4). In contrast, the classic phosphorylation sites recognized by different protein kinases including c-AMP-dependent protein kinase and protein kinase C (reviewed in Kemp and Pearson, (1990) Trends in Biochem. Sci. 15: 342-346), could not be clearly identified. In addition, the proposed motif (Jackson and Peterson, (1990) The EMBO J. 9: 3153-3162) for retention of certain transmembrane proteins in the endoplasmic reticulum as well as the motif

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(Petrou et al., (1993) Trends in Biochem. Sci. 18:41-42) for the fatty acid binding domain of certain intracellular lipid binding proteins could not be identified.

Tissue Distribution of ACAT K₁ Gene Transcripts

The human tissue distribution of K₁ gene transcripts was examined using ³²P-cDNA C1 as the probe. The results (not shown) indicate that it cross-hybridized with poly(A)⁺ RNAs of various discrete sizes, with strong signals at approx. 3 and 4 kb and with weak signals at approx. 4.7 and 7.4 kb. While the intensities varied, these signals were found in poly (A)⁺ RNAs of virtually all of the tissues reported here.

Example V. Homology With Other Enzymes

Protein homology analysis shows that the entire predicted ACAT K₁ protein sequence shares a 48% homology with human fatty acid ligase (Abe et al., (1992) J. Biochem. 111:123-129). In addition, further analysis shows that the predicted K₁ protein contains five separate stretches of linear sequences (TNLIEKSASLDNGGCALTT, GRLVLEFSLLSYAF, GFGPTY, GYVAMKFAQVFGCF, and ARVLVLCVFNSILPGVL, underlined in the protein coding region of Fig. 9, and shown in Sequence Listing Nos. 5 - 9, respectively,) which share significant homology (42%, 57%, 80%, 57%, and 58% respectively, based on firefly luciferase sequences) with the newly identified "signature sequences" (Babbitt et al., (1992) Biochemistry 31: 5594-5604). These signature sequences include three separate segments of peptides and are present in at least twelve different enzymes including firefly luciferase and fatty acid ligase. These enzymes participate in various metabolic functions, and show one common feature--all are involved in the catalysis of acyl adenylate formation followed by acyl thioester formation and subsequent acyl transfer. This analysis suggests that these enzymes share common catalytic mechanisms, and these "signature sequences" constitute part(s) of the active site(s) of these enzymes. Within the ACAT K₁ protein sequence, two different stretches of peptides share homology with the "signature sequence" region #1 (amino acids 193-212 of luciferase), one stretch of peptides shares homology with signature sequence region #2 (amino acids 338-344 of luciferase), while two other stretches of peptides shared homology with the "signature sequence" region #3 (amino acids 338-401 of luciferase).

This finding is important for at least two reasons. First, it suggests that, in addition to functioning as a fatty acyl coenzyme A:cholesterol acyltransferase, the ACAT enzyme may also possess enzymatic activity mechanistically very similar to that of fatty acid: coenzyme A ligase, as well as those of the other enzymes listed in Table 1 of the Babbitt et al. article, supra. Second, this information provides an important clue for designing specific ACAT inhibitors based on known catalytic mechanisms utilized by these enzymes. For example, it should now be possible to design specific ACAT inhibitors based on structural characteristics of various inhibitors already known to inhibit the active site(s) of any of the enzymes listed in Table 1 of the Babbitt, et al. article, supra.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: ACYL COENZYME A: CHOLESTEROL
ACYLTRANSFERASE (ACAT)

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: ASCII Text

(v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: PCT/US93/09704
- (B) FILING DATE: October 12, 1993
- (C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NO.: U.S. SER. NO. 959,950
- (B) FILING DATE: October 14, 1992
- (C) APPLICATION NO.: U.S. SER. NO. 121,057
- (D) FILING DATE: September 10, 1993

(viii) ATTORNEY/AGENT INFORMATION:

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- (B) REGISTRATION NUMBER: 34,858
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(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (617) 227-2700
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 996 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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TAGATAATGG TGGGTGCGCT CTCACAACCT TTTCTGTTCT TGAAGGAGAG AAAACAACC240
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4079 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4011 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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| | |
|---|------|
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| ATA AAA CAG TTG ATA GCA AAG AAG ATA AAG TTG ACA GCA GAG GCA GAG | 1573 |
| Ile Lys Gln Leu Ile Ala Lys Lys Ile Lys Leu Thr Ala Glu Ala Glu | |
| 45 50 55 | |
| GAA TTG AAG CCA TTT TTT ATG AAG GAA GTT GGC AGT CAC TTT GAT GAT | 1621 |
| Glu Leu Lys Pro Phe Phe Met Lys Glu Val Gly Ser His Phe Asp Asp | |
| 60 65 70 75 | |
| TTT GTG ACC AAT CTC ATT GAA AAG TCA GCA TCA TTA GAT AAT GGT GGG | 1669 |
| Phe Val Thr Asn Leu Ile Glu Lys Ser Ala Ser Leu Asp Asn Gly Gly | |
| 80 85 90 | |
| TGC GCT CTC ACA ACC TTT TCT GTT CTT GAA GGA GAG AAA AAC AAC CAT | 1717 |
| Cys Ala Leu Thr Thr Phe Ser Val Leu Glu Gly Glu Lys Asn Asn His | |
| 95 100 105 | |
| AGA GCG AAG GAT TTG AGA GCA CCT CCA GAA CAA GGA AAG ATT TTT ATT | 1765 |
| Arg Ala Lys Asp Leu Arg Ala Pro Pro Glu Gln Gly Lys Ile Phe Ile | |
| 110 115 120 | |
| GCA AGG CGC TCT CTC TTA GAT GAA CTG CTT GAA GTG GAC CAC ATC AGA | 1813 |
| Ala Arg Arg Ser Leu Leu Asp Glu Leu Leu Glu Val Asp His Ile Arg | |
| 125 130 135 | |
| ACA ATA TAT CAC ATG TTT ATT GCC CTC CTC ATT CTC TTT ATC CTC AGC | 1861 |
| Thr Ile Tyr His Met Phe Ile Ala Leu Leu Ile Leu Phe Ile Leu Ser | |
| 140 145 150 155 | |
| ACA CTT GTA GTA GAT TAC ATT GAT GAA GGA AGG CTG GTG CTT GAG TTC | 1909 |
| Thr Leu Val Val Asp Tyr Ile Asp Glu Gly Arg Leu Val Leu Glu Phe | |
| 160 165 170 | |
| AGC CTC CTG TCT TAT GCT TTT GGC AAA TTT CCT ACC GTT GTT TGG ACC | 1957 |
| Ser Leu Leu Ser Tyr Ala Phe Gly Lys Phe Pro Thr Val Val Trp Thr | |
| 175 180 185 | |
| TGG TGG ATC ATG TTC CTG TCT ACA TTT TCA GTT CCC TAT TTT CTG TTT | 2005 |
| Trp Trp Ile Met Phe Leu Ser Thr Phe Ser Val Pro Tyr Phe Leu Phe | |
| 190 195 200 | |

| | |
|---|------|
| CAA CAT TGG CGC ACT GGC TAT AGC AAG AGT TCT CAT CCG CTG ATC CGT Gln His Trp Arg Thr Gly Tyr Ser Lys Ser Ser His Pro Leu Ile Arg 205 210 215 | 2053 |
| TCT CTC TTC CAT GGC TTT CTT TTC ATG ATC TTC CAG ATT GGA GTT CTA Ser Leu Phe His Gly Phe Leu Phe Met Ile Phe Gln Ile Gly Val Leu 220 225 230 235 | 2101 |
| GGT TTT GGA CCA ACA TAT GTT GTG TTA GCA TAT ACA CTG CCA CCA GCT Gly Phe Gly Pro Thr Tyr Val Val Leu Ala Tyr Thr Leu Pro Pro Ala 240 245 250 | 2149 |
| TCC CGG TTC ATC ATT ATA TTC GAG CAG ATT CGT TTT GTA ATG AAG GCC Ser Arg Phe Ile Ile Ile Phe Glu Gln Ile Arg Phe Val Met Lys Ala 255 260 265 | 2197 |
| CAC TCA TTT GTC AGA GAG AAC GTG CCT CGG GTA CTA AAT TCA GCT AAG His Ser Phe Val Arg Glu Asn Val Pro Arg Val Leu Asn Ser Ala Lys 270 275 280 | 2245 |
| GAG AAA TCA AGC ACT GTT CCA ATA CCT ACA GTC AAC CAG TAT TTG TAC Glu Lys Ser Ser Thr Val Pro Ile Pro Thr Val Asn Gln Tyr Leu Tyr 285 290 295 | 2293 |
| TTC TTA TTT GCT CCT ACC CTT ATC TAC CGT GAC AGC TAT CCC AGG AAT Phe Leu Phe Ala Pro Thr Leu Ile Tyr Arg Asp Ser Tyr Pro Arg Asn 300 305 310 315 | 2341 |
| CCC ACT GTA AGA TGG GGT TAT GTC GCT ATG AAG TTT GCA CAG GTC TTT Pro Thr Val Arg Trp Gly Tyr Val Ala Met Lys Phe Ala Gln Val Phe 320 325 330 | 2389 |
| GGT TGC TTT TTC TAT GTG TAC TAC ATC TTT GAA AGG CTT TGT GCC CCC Gly Cys Phe Phe Tyr Val Tyr Tyr Ile Phe Glu Arg Leu Cys Ala Pro 335 340 345 | 2437 |
| TTG TTT CGG AAT ATC AAA CAG GAG CCC TTC AGC GCT CGT GTT CTG GTC Leu Phe Arg Asn Ile Lys Gln Glu Pro Phe Ser Ala Arg Val Leu Val 350 355 360 | 2485 |
| CTA TGT GTA TTT AAC TCC ATC TTG CCA GGT GTG CTG ATT CTC TTC CTT Leu Cys Val Phe Asn Ser Ile Leu Pro Gly Val Leu Ile Leu Phe Leu 365 370 375 | 2533 |
| ACT TTT TTT GCC TTT TTG CAC TGC TGG CTC AAT GCC TTT GCT GAG ATG Thr Phe Phe Ala Phe Leu His Cys Trp Leu Asn Ala Phe Ala Glu Met 380 385 390 395 | 2581 |
| TTA CGC TTT GGT GAC AGG ATG TTC TAT AAG GAT TGG TGG AAC TCC ACG Leu Arg Phe Gly Asp Arg Met Phe Tyr Lys Asp Trp Trp Asn Ser Thr 400 405 410 | 2629 |
| TCA TAC TCC AAC TAT TAT AGA ACC TGG AAT GTG GTG GTC CAT GAC TGG Ser Tyr Ser Asn Tyr Tyr Arg Thr Trp Asn Val Val Val His Asp Trp 415 420 425 | 2677 |

| | |
|---|------|
| CTA TAT TAC TAT GCT TAC AAG GAC TTT CTC TGG TTT TTC TCC AAG AGA Leu Tyr Tyr Tyr Ala Tyr Lys Asp Phe Leu Trp Phe Phe Ser Lys Arg 430 435 440 | 2725 |
| TTC AAA TCT GCT GCC ATG TTA GCT GTC TTT GCT GTA TCT GCT GTA GTA Phe Lys Ser Ala Ala Met Leu Ala Val Phe Ala Val Ser Ala Val Val 445 450 455 | 2773 |
| CAC GAA TAT GCC TTG GCT GTT TGC TTG AGC TTT TTC TAT CCC GTG CTG His Glu Tyr Ala Leu Ala Val Cys Leu Ser Phe Phe Tyr Pro Val Leu 460 465 470 475 | 2821 |
| TTC GTG CTC TTC ATG TTC TTT GGA ATG GCT TTC AAC TTC ATT GTC AAT Phe Val Leu Phe Met Phe Phe Gly Met Ala Phe Asn Phe Ile Val Asn 480 485 490 | 2869 |
| GAT AGT CGG AAA AAG CCG ATT TGG AAT GTT CTG ATG TGG ACT TCT CTT Asp Ser Arg Lys Lys Pro Ile Trp Asn Val Leu Met Trp Thr Ser Leu 495 500 505 | 2917 |
| TTC TTG GGC AAT GGA GTC TTA CTC TGC TTT TAT TCT CAA GAA TGG TAT Phe Leu Gly Asn Gly Val Leu Leu Cys Phe Tyr Ser Gln Glu Trp Tyr 510 515 520 | 2965 |
| GCA CGT CGG CAC TGT CCT CTG AAA AAT CCC ACA TTT TTG GAT TAT GTC Ala Arg Arg His Cys Pro Leu Lys Asn Pro Thr Phe Leu Asp Tyr Val 525 530 535 | 3013 |
| CGG CCA CGT TCC TGG ACT TGT CGT TAC GTG TTT TAGAAGCTTG GACTTTGTTT Arg Pro Arg Ser Trp Thr Cys Arg Tyr Val Phe 540 545 550 | 3066 |
| CCTCCTTGTC ACTGAAGATT GGGTAGCTCC CTGATTGGA GCCAGCTGTT TCCAGTTGTT | 3126 |
| ACTGAAGTTA TCTGTGTTAT TTGGACCACT CCAGGCTTTA CAGATGACTC ACTCCATTCC | 3186 |
| TAGGTCACCT GAAGCCAAAC TGTTGGAAGT TCACTGGAGT CTTGTACACT TAAGCAGAGC | 3246 |
| AGAACTTTTT TTGTGGGGCT GGGTGGGGGG AGAAGACCGA CTAACAGCTG AAGTAATGAC | 3306 |
| AGATTGTTGC TGGGTCATAT CAGCTTTATC CCTGGGTAAT TATATCTGTT TTGTTTCTTG | 3366 |
| ACTCTGTCCA ATCAGAGAAT AAACATCATA GTTCTTGGC CACTGAATTA GCCAAAACAC | 3426 |
| TTAGGAAGAA ATCACTTAAA TACCTCTGGC TTAGAAATTT TTTCATGCAC ACTGTTGGAA | 3486 |
| TGTATGCTAA TTGAACATGC AATTGGGGAA GAAAAAATGT AGAATGATTT TTGCTATTTT | 3546 |
| TAGTAGAAAG AAAATGTCTG TTTTCCAAAG ATAATGTTAT ACATCCTATT TTGTAATTTT | 3606 |
| TTTGAAAAAA GTTCAATGTT CAGTTTTCTT TAGTTTTTAC CTTGTTTTCT CTATAGGTCA | 3666 |
| TGATTTCTGT GAAGCAAAAA GATGCCTTTT ACCATGAATT CTTGAGTTTA CATCAATAAT | 3726 |
| ATTGTATATT AAGGGGATCA GAAGTAGGAA GGAAAAATA AGAGATAGCA GAGGAAAAAG | 3786 |

| | |
|--|------|
| AAAAACATTT CCTCTTATAA CTTCTGAAGT AATTTGTAAA AAAGATTTGT AGAGTCAATC | 3846 |
| ATGTGTTTAA ATTATTTTAT CACAAACTTA ACATGGAAGA TATTCCTTTT TAACTTTGTG | 3906 |
| GTAACCTTCTT TGAAGTTATT TAGAAATATC CTTTGGAACA ATTATTTTAT TGTCTAATAA | 3966 |
| ATATTGACTT CTCTTGAATT ATTTTGCAGA CTAGTGAGTC TGTAC | 4011 |

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 550 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Val Gly Glu Glu Lys Met Ser Leu Arg Asn Arg Leu Ser Lys Ser
 1             5             10             15

Arg Glu Asn Pro Glu Glu Asp Glu Asp Gln Arg Asn Pro Ala Lys Glu
          20             25             30

Ser Leu Glu Thr Pro Ser Asn Gly Arg Ile Asp Ile Lys Gln Leu Ile
          35             40             45

Ala Lys Lys Ile Lys Leu Thr Ala Glu Ala Glu Glu Leu Lys Pro Phe
          50             55             60

Phe Met Lys Glu Val Gly Ser His Phe Asp Asp Phe Val Thr Asn Leu
          65             70             75             80

Ile Glu Lys Ser Ala Ser Leu Asp Asn Gly Gly Cys Ala Leu Thr Thr
          85             90             95

Phe Ser Val Leu Glu Gly Glu Lys Asn Asn His Arg Ala Lys Asp Leu
          100            105            110

Arg Ala Pro Pro Glu Gln Gly Lys Ile Phe Ile Ala Arg Arg Ser Leu
          115            120            125

Leu Asp Glu Leu Leu Glu Val Asp His Ile Arg Thr Ile Tyr His Met
          130            135            140

Phe Ile Ala Leu Leu Ile Leu Phe Ile Leu Ser Thr Leu Val Val Asp
          145            150            155            160

Tyr Ile Asp Glu Gly Arg Leu Val Leu Glu Phe Ser Leu Leu Ser Tyr
          165            170            175

Ala Phe Gly Lys Phe Pro Thr Val Val Trp Thr Trp Trp Ile Met Phe
          180            185            190

Leu Ser Thr Phe Ser Val Pro Tyr Phe Leu Phe Gln His Trp Arg Thr
          195            200            205

Gly Tyr Ser Lys Ser Ser His Pro Leu Ile Arg Ser Leu Phe His Gly
          210            215            220

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Phe Leu Phe Met Ile Phe Gln Ile Gly Val Leu Gly Phe Gly Pro Thr
 225 230 235 240
 Tyr Val Val Leu Ala Tyr Thr Leu Pro Pro Ala Ser Arg Phe Ile Ile
 245 250 255
 Ile Phe Glu Gln Ile Arg Phe Val Met Lys Ala His Ser Phe Val Arg
 260 265 270
 Glu Asn Val Pro Arg Val Leu Asn Ser Ala Lys Glu Lys Ser Ser Thr
 275 280 285
 Val Pro Ile Pro Thr Val Asn Gln Tyr Leu Tyr Phe Leu Phe Ala Pro
 290 295 300
 Thr Leu Ile Tyr Arg Asp Ser Tyr Pro Arg Asn Pro Thr Val Arg Trp
 305 310 315 320
 Gly Tyr Val Ala Met Lys Phe Ala Gln Val Phe Gly Cys Phe Phe Tyr
 325 330 335
 Val Tyr Tyr Ile Phe Glu Arg Leu Cys Ala Pro Leu Phe Arg Asn Ile
 340 345 350
 Lys Gln Glu Pro Phe Ser Ala Arg Val Leu Val Leu Cys Val Phe Asn
 355 360 365
 Ser Ile Leu Pro Gly Val Leu Ile Leu Phe Leu Thr Phe Phe Ala Phe
 370 375 380
 Leu His Cys Trp Leu Asn Ala Phe Ala Glu Met Leu Arg Phe Gly Asp
 385 390 395 400
 Arg Met Phe Tyr Lys Asp Trp Trp Asn Ser Thr Ser Tyr Ser Asn Tyr
 405 410 415
 Tyr Arg Thr Trp Asn Val Val Val His Asp Trp Leu Tyr Tyr Tyr Ala
 420 425 430
 Tyr Lys Asp Phe Leu Trp Phe Phe Ser Lys Arg Phe Lys Ser Ala Ala
 435 440 445
 Met Leu Ala Val Phe Ala Val Ser Ala Val Val His Glu Tyr Ala Leu
 450 455 460
 Ala Val Cys Leu Ser Phe Phe Tyr Pro Val Leu Phe Val Leu Phe Met
 465 470 475 480
 Phe Phe Gly Met Ala Phe Asn Phe Ile Val Asn Asp Ser Arg Lys Lys
 485 490 495
 Pro Ile Trp Asn Val Leu Met Trp Thr Ser Leu Phe Leu Gly Asn Gly
 500 505 510

-33-

Val Leu Leu Cys Phe Tyr Ser Gln Glu Trp Tyr Ala Arg Arg His Cys
 515 520 525

Pro Leu Lys Asn Pro Thr Phe Leu Asp Tyr Val Arg Pro Arg Ser Trp
 530 535 540

Thr Cys Arg Tyr Val Phe
 545 550

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Asn Leu Ile Glu Lys Ser Ala Ser Leu Asp Asn Gly Gly Cys Ala
 1 5 10 15

Leu Thr Thr

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Arg Leu Val Leu Glu Phe Ser Leu Leu Ser Tyr Ala Phe
 1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Phe Gly Pro Thr Tyr
 1 5

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(2) INFORMATION FOR SEQ ID NO:8

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8

Gly Tyr Val Ala Met Lys Phe Ala Gln Val Phe Gly Cys Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:9

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 9:

Ala Arg Val Leu Val Leu Cys Val Phe Asn Ser Ile Leu Pro Gly Val
1 5 10 15

Leu

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WHAT IS CLAIMED IS:

1. Isolated, biologically active acyl coenzyme A:cholesterol acyltransferase, or a biologically active fragment thereof.
2. Biologically active acyl coenzyme A:cholesterol acyltransferase of claim 1 which is of human origin.
3. The biologically active acyl coenzyme A:cholesterol acyltransferase of claim 1 produced by recombinant DNA techniques from the nucleic acid sequence selected from the group consisting of the nucleotide sequences shown in Sequence Listing No. 1, 2, and 3, their functional equivalents and fragments thereof.
4. The biologically active acyl coenzyme A:cholesterol acyltransferase of claim 1 having an amino acid sequence containing a linear sequence selected from the group consisting of TNLIEKSASLDNGGCALTT, GRLVLEFSLLSYAF, GFGPTY, GYVAMKFAQVFGCF, and ARVLVLCVFNSILPGVL, as shown in Sequence Listing Nos. 5-9, respectively, and their functional equivalents.
5. The biologically active acyl coenzyme A:cholesterol acyltransferase of claim 4 wherein the selected linear sequence is catalytically active.
6. The biologically active acyl coenzyme A: cholesterol acyltransferase of claim 1 produced by recombinant DNA techniques from the nucleic acid sequence shown in Sequence Listing No. 3.
7. Biologically active coenzyme A: cholesterol acyltransferase having the amino acid sequence shown in Sequence Listing No. 4 and functional equivalents thereof.
8. Isolated nucleic acid encoding biologically active acyl coenzyme A:cholesterol acyltransferase, or the functional equivalent of said nucleic acid.
9. The nucleic acid of claim 8, wherein said nucleic acid is DNA.

10. The nucleic acid of claim 8, wherein said nucleic acid is human.
11. An expression vector containing nucleic acid encoding the biologically active human acyl coenzyme A:cholesterol acyltransferase.
12. The expression vector of claim 11 wherein the nucleic acid is DNA.
13. The expression vector of claim 11 wherein the nucleic acid comprises the sequence selected from the group consisting of the sequences shown in Sequence Listing Nos. 1, 2 and 3.
14. A host cell transformed with the expression vector of claim 11.
15. A non-human cell which either lacks or has deficient levels of endogenous acyl coenzyme A:cholesterol acyltransferase activity, said cell being transformed with a nucleic acid encoding human acyl coenzyme A:cholesterol acyltransferase such that the cell produces an excess of cholesterol esters causing the formation of detectable, cytoplasmic lipid droplets.
16. The cell of claim 15 wherein the cytoplasmic lipid droplets are visibly detectable.
17. The cell of claim 15 wherein the cell is a 14e cell.
18. A method of testing for an agent capable of specifically inhibiting human acyl coenzyme A:cholesterol acyltransferase, the method comprising the steps of
 - exposing the cell of claim 15 to the agent under conditions which allow the agent to be taken up into the cell, and
 - examining the exposed cell for substantial disappearance of the lipid droplets, wherein substantial disappearance indicates inhibition of human acyl coenzyme A:cholesterol transferase.
19. A method of selectively inhibiting synthesis of acyl coenzyme A:cholesterol acyltransferase (ACAT) without substantially inhibiting the synthesis of non-targeted enzymes, the method comprising the step of:

introducing into a cell a stable, single-stranded oligonucleotide having a nucleotide sequence substantially complementary to at least a portion of the nucleotide sequence of messenger ribonucleic acid (mRNA) encoding ACAT, such that said stable oligonucleotide hybridizes with said mRNA to substantially block the expression of ACAT.

20. The method of claim 19 wherein the selective inhibition of synthesis of acyl coenzyme A:cholesterol acyltransferase occurs in vivo.

21. The method of claim 19 wherein the selective inhibition of synthesis of acyl coenzyme A:cholesterol acyltransferase occurs in vitro.

22. A method of selectively inhibiting synthesis of the enzyme acyl coenzyme A:cholesterol acyltransferase (ACAT) without substantially inhibiting the synthesis of non-targeted enzymes, the method comprising the step of:

introducing into a cell a stable antisense oligonucleotide having a nucleotide sequence substantially complementary to the gene encoding ACAT, such that said stable antisense oligonucleotide hybridizes with said DNA to substantially block expression of ACAT.

23. The method of claim 22 wherein the selective inhibition of synthesis of acyl coenzyme A:cholesterol acyltransferase occurs in vivo.

24. The method of claim 22 wherein the selective inhibition of synthesis of acyl coenzyme A:cholesterol acyltransferase occurs in vitro.

25. A probe for nucleic acid encoding acyl coenzyme A:cholesterol acyltransferase, comprising a labeled single stranded nucleic acid encoding at least a portion of the enzyme acyl coenzyme A:cholesterol acyltransferase, or the functional equivalent of said nucleic acid.

26. A probe of claim 25 wherein the single stranded nucleic acid is DNA.

27. A primer for polymerase-mediated replication of a nucleotide sequence encoding acyl coenzyme A:cholesterol acyltransferase, comprising a single stranded nucleic acid substantially complementary to at least a portion of the nucleotide sequence encoding acyl coenzyme A:cholesterol acyltransferase.

28. A transgenic animal having cells which contain a nucleotide sequence encoding an antisense oligonucleotide which hybridizes to the transcript of the gene encoding acyl coenzyme A:cholesterol acyltransferase, to substantially block expression of enzyme acyl coenzyme A:cholesterol acyltransferase.

29. The transgenic animal of claim 28 selected from the group consisting of mice, rats, rabbits, chickens, cows, and pigs.

30. The transgenic animal of claim 28 wherein the animal has reduced body cholesterol levels.

31. A transgenic animal having cells which contain and stably express a nucleotide sequence encoding human acyl coenzyme A:cholesterol acyltransferase, or an active portion thereof.

32. The transgenic animal of claim 31 selected from the group consisting of mice, rates, rabbits, chickens, cows and pigs.

33. A mammalian germ cell containing a gene encoding an oligonucleotide antisense transcript which hybridizes to the transcript of the gene encoding acyl coenzyme A: cholesterol acyltransferase to substantially block expression of acyl coenzyme A: cholesterol acyltransferase.

34. A method of testing for an agent capable of specifically inhibiting human acyl coenzyme A: cholesterol acyltransferase, the method comprising the steps of:

exposing the agent to a non-human cell which either lacks or has deficient levels of endogenous acyl coenzyme A: cholesterol acyltransferase activity, said cell being stably transfected with a nucleic acid encoding human acyl coenzyme A: cholesterol acyltransferase.

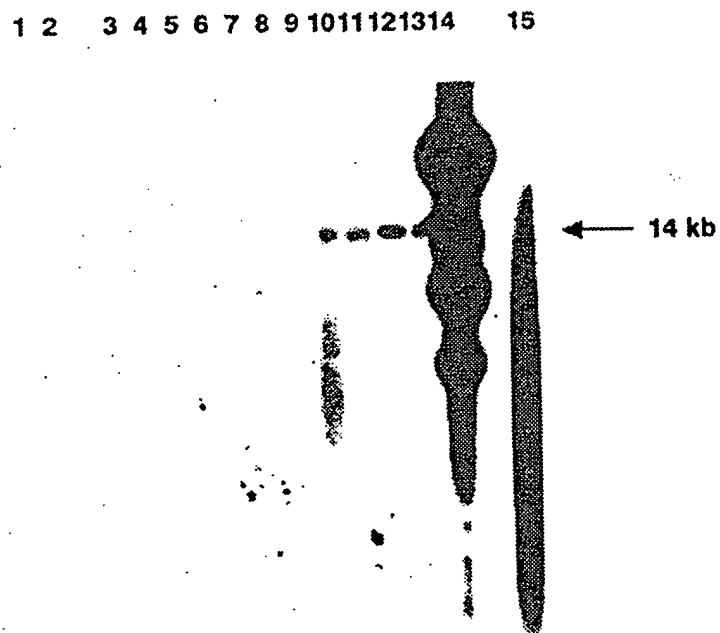
inspecting said for acyl coenzyme A: cholesterol acyltransferase inhibition.

35. The method of claim 30 wherein the agent is selected from the group consisting of small organic molecules, antisense DNA, and antisense RNA.

36. The method of claim 30 wherein the non-human cell is selected from the group consisting of T2-4, T2-8, and T2-10.

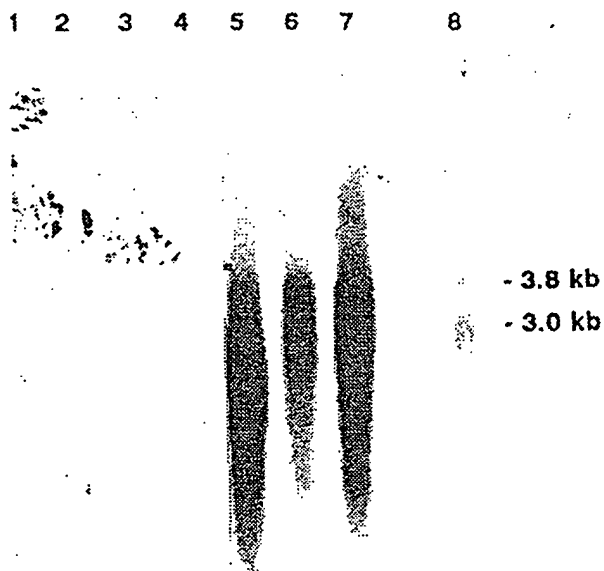
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FIG. 1



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FIG.2



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FIG. 3

The Nucleotide Sequence of cDNA C₁

gaaaccctgcaaaggagtcacctagagacacctagtaatgggtcgaattgacataaaacagt
tgatagcaaagaagataaagttgacagcagaggcagaggaattgaagccattttttatga
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FIG. 4

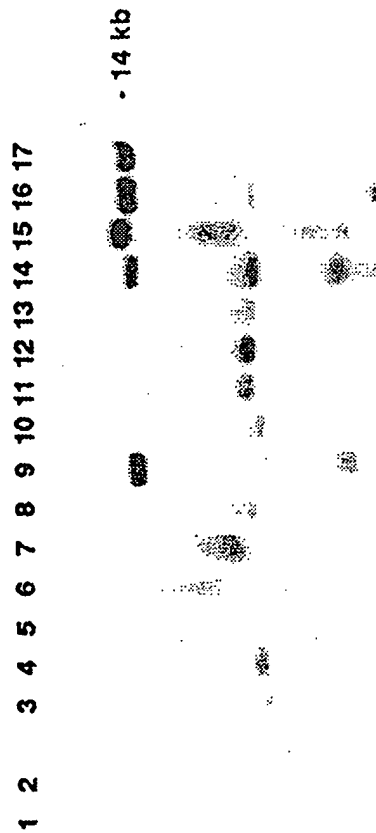


FIG.5A

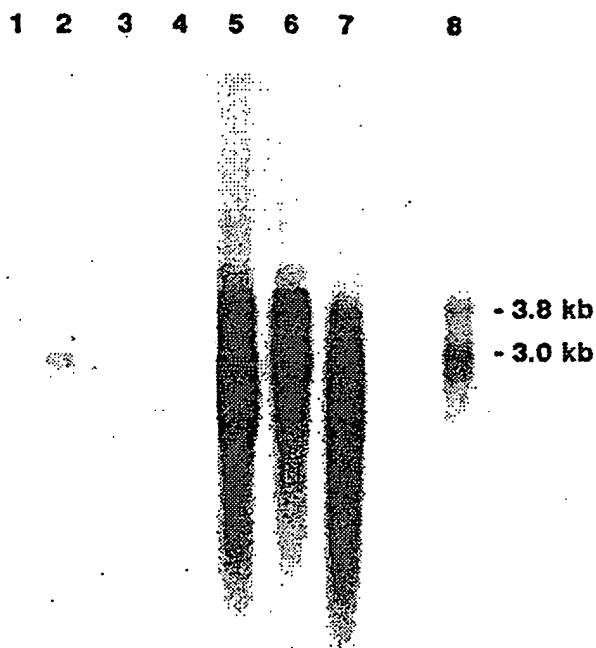


FIG.5B

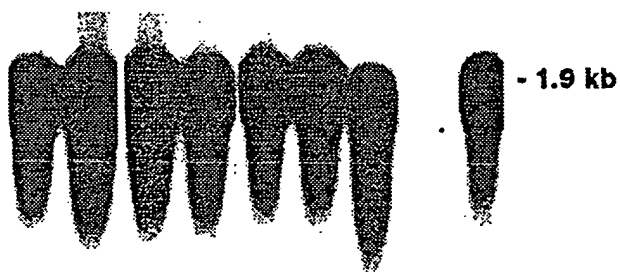


FIG. 6

gggtagagacgggggtttcaccgtgttagccaggatgggtctggatctcctgacctcgtgatccac
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ccttttcaaaagtcagaccctaggaagctggagggaggtggggcatgggttttacagtgaatttc
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gagcagatcgttttgtaatgaaggccactcatttgtagagagaaacgtgcctcgggtactaat
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tgctatgaagtttgacaggtctttggttgctttttctatgtgtactacatctttgaaaggctt
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FIG. 6 CONT.'

tatttaactccatcttgccaggtgtgctgattctcttccctactttttttgcctttttgcactg
ctggctcaatgcctttgctgagatgttacgctttgggtgacaggatgttctataaggattgggtgg
aactccacgtcatactccaactattatagaacctggaatgtgggtggccatgactggctatatt
actatgcttacaaggactttctctgggttttctccaagagattcaaactctgctgccatgttagc
tgtctttgctgtatctgctgtagtacacgaatatgccttggtgtttgcttgagctttttctat
cccgtgctgttcgtgctcttcatgttctttggaatggctttcaacttcattgtcaatgatagtc
ggaaaaagccgattttggaatgttctgatgtggacttctcttttcttgggcaatggagcttact
ctgctttttattctcaagaatgggtatgcacgtcggcactgtacctctgaaaaatcccacattttt
ggattatgtcctgtcacgttcctggacttgtcgttacgtgttttagaagcttggactttgtttc
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tggggctgggtgggggggagaagaccgactaacagctgaagtaatgacagattgttgctgggtca
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agcagaggaaaaaagaaaaacatttctcttataacttctgaagtaattttgtaaaaaagatttgt
agagtcaatcatgtgtttaaattattttatcacaaacttaacatggaagatatccttttttaac
tttggtggaacttctttgaagttattttagaatatcctttggaacaattattttattgtcta
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FIG.7A

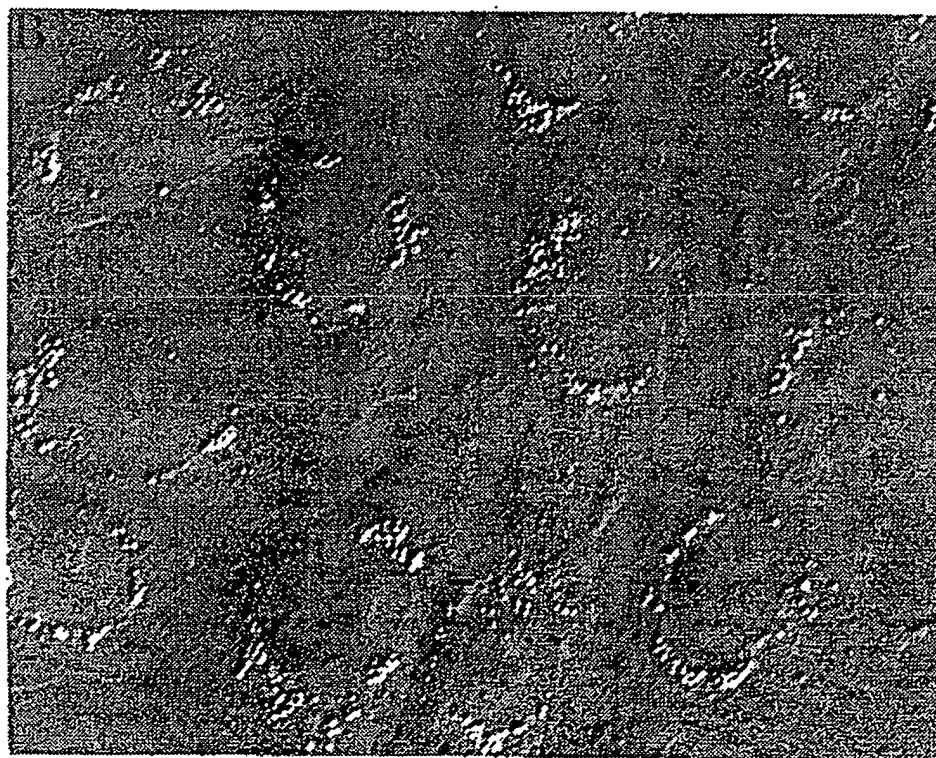
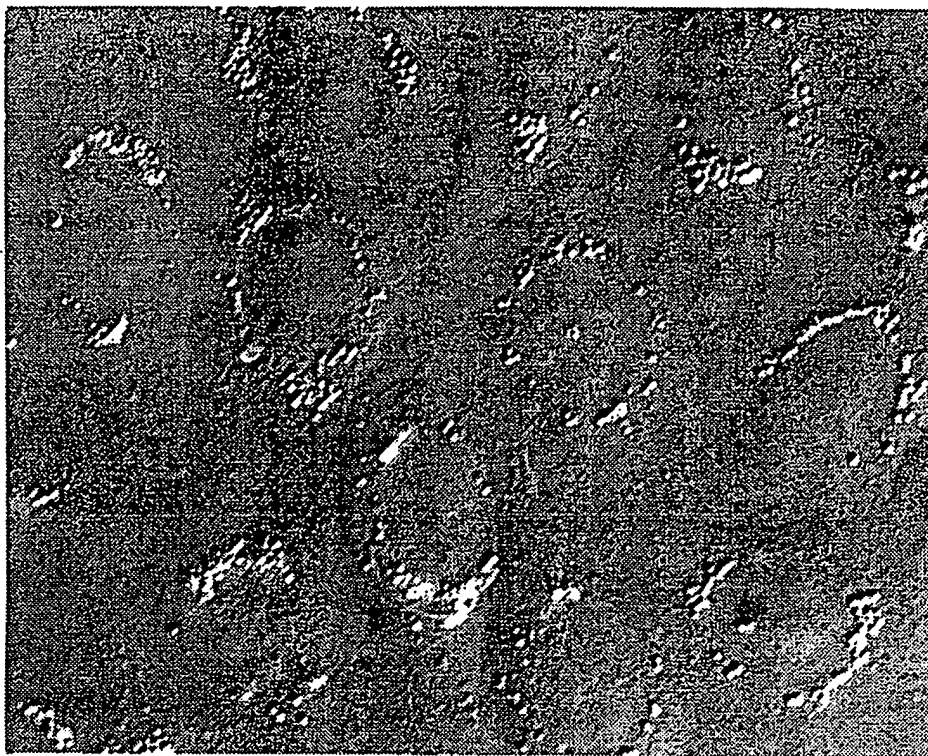


FIG.7B

FIG. 7C

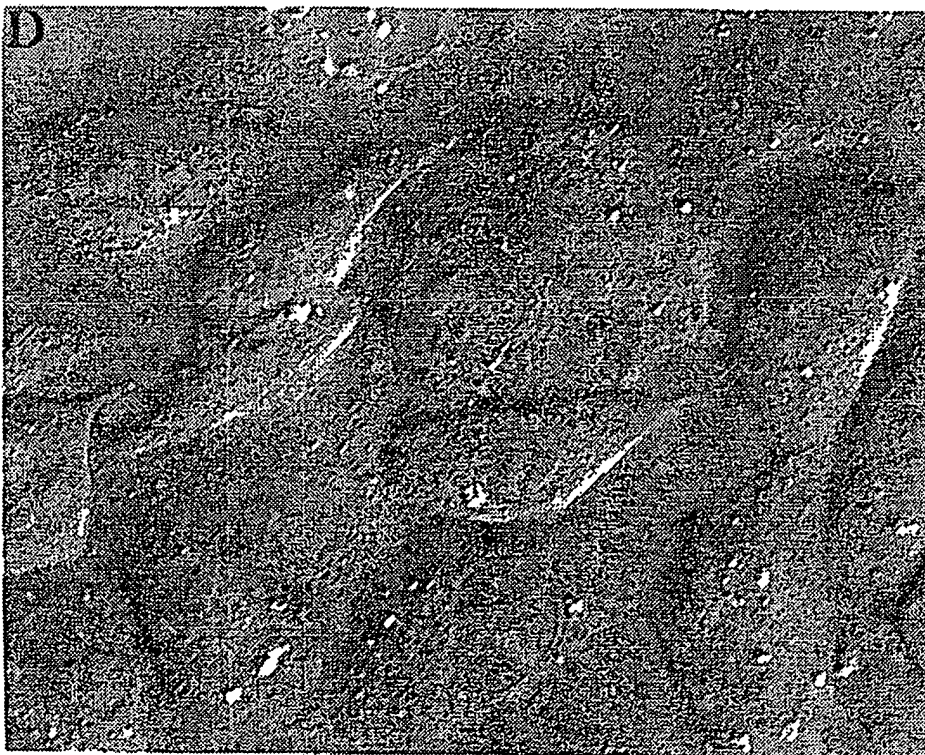


FIG. 7D

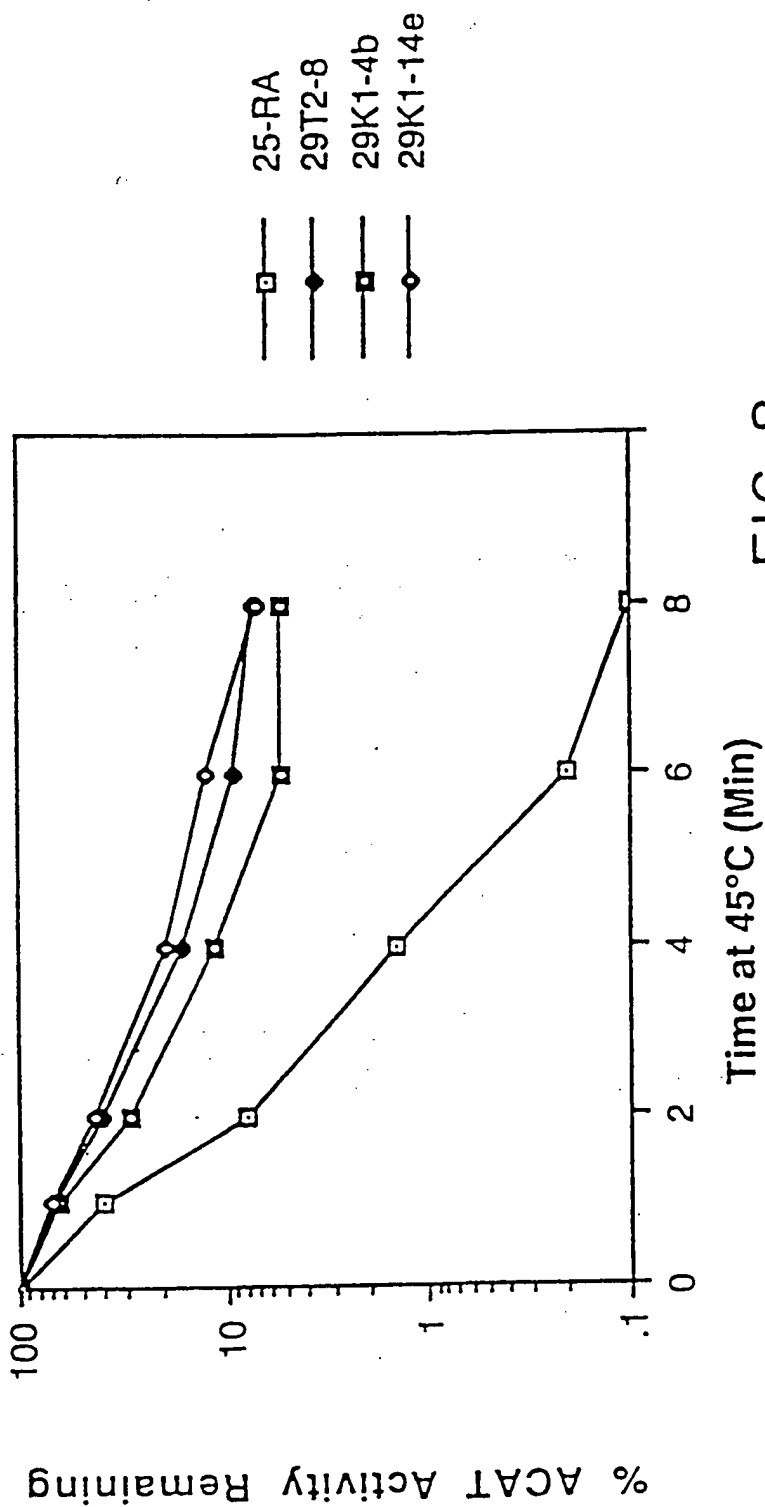


FIG. 8

[illegible]

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FIG. 9 **CON'T.**

Nucleotide and Predicted Amino Acid Sequences of Human ACAT cDNA K1

| | | | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| 1 | ATG | GTG | GGT | GAA | GAG | GAG | ATG | ATG | TCT | CTA | AGA | AAC | CGG | CTG | TCA | AAG | TCC | AGG | GAA | AAT | CCT | 1456 |
| 21 | E | D | E | D | E | D | E | D | E | D | E | D | E | D | E | D | E | D | E | D | E | 1516 |
| 41 | R | I | D | I | K | Q | L* | I | A | G | A | G | G | G | A | G | A | G | A | G | A | 1576 |
| 61 | L* | K | P | F | M | K | E | V | G | S | G | G | G | G | G | G | G | G | G | G | G | 1636 |
| 81 | I | E | K | S | A | S | L* | D | N | G | A | A | T | G | A | T | G | A | T | G | A | 1696 |
| 101 | E | G | E | K | N | N | H | R | C | T | C | T | C | T | C | T | C | T | C | T | C | 1756 |
| 121 | I | F | I | A | R | R | A | T | T | T | G | C | C | C | C | C | C | C | C | C | C | 1816 |
| 141 | I | Y | H | M | F | I | A | G | G | G | G | G | G | G | G | G | G | G | G | G | G | 1876 |
| 161 | Y | I | D | E | G | R | L | V | L | V | L | V | L | V | L | V | L | V | L | V | L | 1936 |
| 181 | F | P | T | T | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | 1996 |
| 201 | F | L | F | Q | H | W | Q | H | W | Q | H | W | Q | H | W | Q | H | W | Q | H | W | 2056 |
| 221 | L | F | H | G | F | L | F | H | G | F | L | F | H | G | F | L | F | H | G | F | L | 2116 |
| 241 | Y | 2 | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | V | 2176 |

FIG. 9